PLGA drug delivery

ELSEVIER

Journal of Controlled Release 41 (1996) 131–145

journal of controlled release

Tetanus toxoid and synthetic malaria antigen containing poly(lactide)/poly(lactide-co-glycolide) microspheres: importance of polymer degradation and antigen release for immune response

Claudio Thomasin[a], Giampietro Corradin[b], Ying Men[b], Hans P. Merkle[a], Bruno Gander[a,*]

[a]*Department of Pharmacy, Swiss Federal Institute of Technology (ETH), Winterthurerstr. 190, 8057 Zürich, Switzerland*

[b]*Institute of Biochemistry, University of Lausanne, Chemin des Boveresses. 155, 1066 Epalinges, Switzerland*

Received 20 May 1995; revised 20 December 1995; accepted 24 January 1996

Abstract

The importance of in vitro degradation of poly(lactide)/poly(lactide-co-glycolide) (PLA/PLGA) microspheres and of the concomitant in vitro release of a natural and a synthetic antigen for eliciting immune response was studied in mice. A variety of PLAs and PLGAs differing in molecular weight ($\bar{M}_r$ of 14–130 kDa) and in polymer composition (lactic/glycolic acid ratio of 100:00, 75:25, and 50:50) were examined for their in vitro degradation, which ranged from approximately 4 to 20 weeks. Three specific polymers were then selected for microencapsulation of the two antigens tetanus toxoid (TT) and a weakly immunogenic synthetic branched malaria peptide (P30B2). The in vitro release data showed that antigen delivery correlates fairly well with polymer degradation giving rise to a distinct burst release during the first 24 h and an additional release pulse towards the end of polymer degradation. After single subcutaneous administration in mice, long lasting high antibody titers were obtained with the antigen containing microspheres, as compared to TT adsorbed on alum or to P30B2 in Incomplete Freund's Adjuvant. Moreover, the immune responses induced by microspheres were clearly influenced by the antigen release kinetics, the polymer type and the size of the microspheres. The results demonstrate the immunopotentiating properties of the biodegradable microspheres and their potential to elicit long-lasting immune responses after single administration when tailoring in vitro release characteristics and particle size.

Keywords: Poly(lactide) (PLA); Poly(lactide-co-glycolide) (PLGA); Biodegradable microspheres; Polymer degradation; Tetanus toxoid; Synthetic malaria antigen; Antigen release; Immune responses

1. Introduction

The search for new immunological adjuvants and antigen delivery systems has attracted the interest of various national and international health institutes and organizations, as well as of academic and industrial research scientists. Three major objectives of practical importance have been defined to improve existing vaccines. Clearly, formulations or delivery systems should (i) mimic booster doses after single parenteral administration, (ii) exhibit adequate immunostimulating properties for weakly immunogenic (synthetic) antigens, and (iii) elicit a strong immune response after nasal or oral administration. In this light, biodegradable microspheres (MS) based on poly(lactide) (PLA) or poly(lactide-co-glycolide) (PLGA) are probably the most promising of all the

*Corresponding author. Tel: +41-1-2576012; Fax: +41-1-2625459.

PII S0168-3659(96)01363-6

antigen delivery systems studied so far. In addition to the immunostimulating properties of most other adjuvant formulations or delivery systems, biodegradable MS also provide prolonged and even pulsatile antigen release over several months, thereby mimicking conventional immunization schedules [1–4]. It is the particular goal of replacing the necessary booster injections of conventional vaccines by a single injection of antigen containing microspheres which has been addressed by the WHO in an ongoing program initiated in 1989 [5,6]. In addition, the demonstrated targeting of biodegradable microspheres to the Peyer's patches of the GI-tract or to the nasal mucosa holds great promise for peroral and nasal immunization [7–10].

The basis for the present interest in PLA/PLGA-based biodegradable microspheres for vaccine delivery was laid undoubtedly in the late 1970s to mid-1980s, particularly by pioneering investigations on (i) single-step immunization by sustained antigen release [11,12], (ii) peptide drugs being released from PLGA-microspheres in a pulsatile manner [13], and on (iii) the biodegradation of and tissue reaction to PLA/PLGA-microspheres [14,15].

Various studies have been conducted to clarify the in vitro and in vivo degradation mechanisms of PLA/PLGA polymers as it became evident that drug release kinetics and pharmacological responses greatly depend on the polymer characteristics and biodegradation behaviour [16–22]. These investigations revealed that in particular the release of macromolecules is closely related to polymer degradation and, hence, to copolymer composition and molecular weight. Moreover, it has been found that the degradation pattern of polymers obtained from different suppliers is also affected by polymer quality, i.e. by the presence of low molecular weight fractions and monomers [23]. Special attention has been paid further to the role of water involved in polymer degradation and its influence on glass transition temperature, T_g [24,25]. On the other side, relatively little knowledge is available at present on the interaction between polymer and protein under release conditions. It has been reported that osmotic effects and ionic interactions between polymer end groups and the amide bond or free amino groups of polypeptides greatly affect the release pattern of proteinaceous compounds from microspheres [26–28]. It has also been recognized for some time, and reemphasized more recently, that during polymer degradation, the hydrolysis products are likely to create an acidic environment inside the microspheres and within their microenvironment, which may severely compromise protein stability in in vitro release studies and also in an in vivo situation [16,28,29]. However, general conclusions about the importance of an acidic microenvironment on protein stability cannot been drawn as every protein forms its own case.

Over the past few years, the particular aspect of antigen delivery from biodegradable MS and its relevance for the immune response has been investigated by several groups. Most of these studies have focused on the natural antigens tetanus toxoid (TT) [1,2,10,30] and diphtheria toxoid (DT) [31], and only very few have considered synthetic antigens [3]. Generally, some correlations have been found between the kinetics of polymer degradation and of antigen release, but not between in vitro release kinetics and immune response. All of these studies confirm the very pronounced immunostimulating properties of microspheres.

Here, we report on the correlation between polymer degradation and antigen release from microspheres, and on the importance of their release kinetics and particle size for immune response. Investigations on two different types of antigens are being summarized, i.e. conventional tetanus toxoid and a synthetic antimalarial antigen.

2. Experimental

2.1. Materials

Lyophilized tetanus toxoid (TT) (approximately 90 Lf/mg protein, Institute Merieux, Lyon, France) was provided by WHO (Geneva, Switzerland). A tetra-branched synthetic peptide composed of an universal T helper epitope from tetanus toxin (947–967) and a B-cell epitope from the repetitive region of *Plasmodium berghei* circumsporozoite protein (P30B2) was synthesized by using the F-moc strategy [32]. The molecular weights of the two antigens are approximately 150 000 for TT and 16 000 for P30B2. Various poly(D,L-lactides) (PLA)

and poly(D,L-lactide-co-glycolides) (PLGA) differing in molecular weight and composition were purchased from Boehringer Ingelheim, Ingelheim, Germany; they included the PLAs Resomer® R202 ($\bar{M}_w$ 14 600), R203 ($\bar{M}_w$ 23 300) and R206 ($\bar{M}_w$ 129 700), the PLGAs 50:50 Resomer® RG502 ($\bar{M}_w$ 13 700) and RG503 ($\bar{M}_w$ 35 100), and the PLGAs 75:25 Resomer® RG752 ($\bar{M}_w$ 17 000) and RG755 ($\bar{M}_w$ 68 600). Hereafter, the following designations will be used to specify the type of polymer: PLA-14 kDa, PLA-23 kDa, PLA-130 kDa, PLGA50:50-14 kDa, PLGA50:50–35 kDa, PLGA75:25-17 kDa and PLGA75:25–69 kDa. All other chemicals used were of analytical grade (from Fluka, Buchs, Switzerland), unless otherwise specified.

2.2. Preparation of microspheres

Empty and antigen loaded microspheres (MS) were prepared by the two microencapsulation techniques, spray-drying (SD) and coacervation (CO), as described in detail elsewhere [33,34]. Briefly, in the spray-drying process, 100 mg of TT or 20 mg of P30B2 were dissolved in 2.0 ml water. The resulting antigen solutions were finely dispersed in 100.0 g (for TT) or 40.0 g (for P30B2) of a 5% (w/w) polymer solution in ethyl formate by means of an ultrasonic processor. The W/O-dispersions were subsequently spray-dried in a laboratory spray-dryer (Model 190, Büchi, Flawil, Switzerland). The microspheres were washed with 0.1% (w/w) Synperonic® F68 solution (ICI, Middlesbrough, UK), collected on a 0.2 μm cellulose acetate membrane filter and dried under vacuum for 24 h.

In the coacervation process, the polymers were dissolved in dichloromethane (DCM) at a concentration of 10% (w/w), wherein the antigen solutions were dispersed, as described above. The dispersion was introduced into a jacketed vessel (250 ml) equipped with baffles and an anchor stirrer. Coacervation was induced by introducing a predetermined necessary amount of silicone oil (DC-200, 1070 mPa/s); with respect to the total mass of the coacervation mixture, the percentage of added silicone oil was 73% for PLA-14 kDa, 70% for PLA-23 kDa, 55% for PLA-130 kDa, 68% for PLGA75:25-17 kDa, 53% for PLGA75:25–69 kDa, 58% for PLGA50:50-14 kDa and 47% for PLGA50:50-35 kDa. Stirring was set at 1000 rev./min and the temperature maintained at 10°C. The stable coacervation dispersion was slowly poured into 1200 ml of hardening agent (octamethylcyclotetrasiloxane, OMCTS) to solidify the microspheres. Stirring was continued for 30 min, and the microspheres collected on a sintered glass filter and washed with 100 ml hexane. The product was then air-dried for 5 min and resuspended three times in 0.1% (w/w) Synperonic® F68 solution. Drying took place under the conditions specified above.

2.3. Polymer molecular weight and in vitro polymer degradation

The polymers used were characterized by gel permeation chromatography (GPC) for molecular weights, $\bar{M}_w$ and $\bar{M}_n$, and polydispersity, P. The polymers were dissolved at a concentration of 0.2% (w/w) in tetrahydrofuran (THF) (purity > 99.5%, stabilized with butylhydroxytoluene, residual water < 0.01%; Scharlau, ETG-Chemie, Tägerig, Switzerland). Toluene was used as internal standard at a concentration of 0.02% (w/w). A 100-μl aliquot of the polymer solution was injected into a 20 μl loop (Model 7125, Rheodyne, Berkley, USA) of a HPLC-system (Pump L 6000, Merck Hitachi, Merck ABS, Dietikon, Switzerland) and separated on a mixed gel column with a molecular weight separation range of 0.2 to 2000 (PL-gel 5 μm, 300 × 7.5 mm^2, Polymer Laboratories, Shropshire, UK). Column calibration was performed with a monodisperse polystyrene kit covering a molecular weight range of 1.32 to 1030 (PL polystyrene-medium molecular weight kit, Polymer Laboratories). THF was used as eluent at a flow rate of 1 ml/min, and the column temperature maintained at 30°C (Column oven, Merck Hitachi). Elution profiles were detected refractometrically (RI-detector, ERC-7512, Erma, Tokyo, Japan) at 30°C and a sensitivity of 4. The data were analyzed by a GPC integrator (D-2520, Merck Hitachi).

The in vitro degradation behavior of unloaded microspheres, prepared by coacervation, was characterized by suspending 50 mg of microspheres in borosilicate vials (Chromacol®, Welwyn Garden City, UK) containing 4.0 ml of PBS pH 7.4, pre-

served with 0.02% (w/w) sodium azide. The dispersions were ultrasonicated to facilitate wetting, and the vials fixed horizontally in a drum rotating at 3 rev./min and 37°C (Drum Roller, TC 1000/3, Brouwer, Lucerne, Switzerland). At defined intervals, the samples were analyzed in triplicate. The content of each vial was centrifuged, the supernatant withdrawn, and its pH measured. The medium of the remaining samples was replaced by fresh buffer to readjust the pH. The isolated microspheres or polymeric mass was dried under vacuum for 2 h and dissolved in THF (0.2%, w/w). The filtered solutions were finally analyzed by GPC.

2.4. Morphology and size of microspheres

For morphological examination, the microspheres were mounted on double-faced adhesive tape, sputtered with platinum and viewed in a Hitachi S-700 scanning electron microscope.

Size and size distribution of microspheres were measured by laser light scattering (Mastersizer X, Malvern, Malvern, UK). Typically, 50–100 mg of microspheres were carefully pounded in a mortar with a few drops of polysorbate 20, followed by the addition of 3 ml water. The particle dispersion was transferred into a small volume presentation unit (Malvern) and ultrasonicated for 30 s at output 40 (Vibra cell, VC50T, Sonics and Materials, Danburry, USA). Calculation of particle sizes from the scattered light was based on Mie's theory accounting for the optical properties of the polymers. The refractive index of PLA and PLGA was calculated from group contributions [35].

2.5. Antigen loading in the microspheres

The antigen content was determined with 20–30 mg of antigen loaded microspheres dissolved in 2 ml dichloromethane; the dispersion was vacuum-filtered on a 0.2 μm regenerated cellulose RC58 membrane filter (Schleicher and Schuell, Dassel, Germany). Filters were washed three times with the same solvent and air dried. The antigens were eluted three times with 3 ml PBS, pH 7.4. The solution was analyzed by Bradford's protein assay (for TT) (Bio-Rad, Munich Germany) or fluorometrically (for P30B2) (Fluoromax®, Spex Industries, Edison, NJ, USA), with excitation and emission wavelengths of 278 nm and 340 nm, respectively.

2.6. In vitro antigen release

Antigen release was determined by suspending 20–100 mg of microspheres in borosilicate vials with screw caps (Chromacol®, Welwyn Garden City, UK) containing 4.0 ml of PBS pH 7.4, preserved with 0.02% sodium azide. The dispersions were ultrasonicated to facilitate wetting, and the vials fixed horizontally in a drum rotating at 3 rev./min at 37°C. At regular intervals, the supernatant from each vial was collected by centrifugation and assayed immediately by Bradford's assay (for TT) or fluorometrically, as specified above. At every time point, 3 ml of the release medium were replaced by fresh medium and the pH readjusted to 7.4.

2.7. Immunogenicity of microencapsulated antigens in mice

Antibody titers of microencapsulated antigens as compared to TT adsorbates on aluminum hydroxide (Alum) or P30B2 in Incomplete Freund's Adjuvant (IFA) were evaluated as described elsewhere [36]. In short, groups of eight BALB/c female mice 8–10 weeks of age (OLAC, Bicester Oxon, UK) received a total dose of either 20 μg (1.7 Lf) of TT or 30 μg of P30B2 in various formulations by subcutaneous injection at the base of the tail. For immunization with tetanus toxoid, five groups of mice received a single injection of TT-MS preparations (SD502, SD752, CO502, CO752 and CO206), and another group received a single injection of TT-alum (Alum-1). Correspondingly, for the immunization with P30B2, five groups of mice received a single injection of P30B2-MS (SD502, SD752, CO502, CO752 and CO206), and another group a single injection of P30B2-IFA (IFA-1). For injection, the microspheres were suspended in 100 μl of 5% (w/w) sterile lecithin solution (Ovothin® 170, Lucas Meyer, Hamburg, Germany). Lecithin was used for dispersing microspheres because of its excellent wetting and suspending properties and for its high biocompatibility and biodegradability. No effect on antibody production was observed when mice were injected with lecithin alone. After immunization,

mouse sera were collected at an interval of 2–4 weeks by tail bleeding. Specific serum antibodies were measured by ELISA, as described elsewhere [36]. The antibody titer was expressed as the reciprocal of the highest positive serum dilution. Student's *t*-test was used on logarithm-transformed data to compare antibody levels obtained from each group at various time points, according to accepted convention [44].

3. Results

Most important characteristics of a vaccine delivery system based on biodegradable polymers encompass the polymer degradation time, the microspheres' size, the antigen release kinetics and the immunological responses. All of these four parameters were investigated in this study and reported below.

3.1. In vitro polymer degradation

For selection of appropriate polymers for antigen delivery, microspheres were prepared from a series of homo- and copolymers of lactic and glycolic acid, varying in composition and molecular weight, and were subjected to an in vitro degradation study. The microspheres were prepared by coacervation and showed a monomodal size distribution in the range of 10 to 100 μm.

First, the effect of polymer composition was examined with three low molecular weight polymers (Fig. 1). Each point represents the mean of three individual samples of microspheres. The degradation profiles appear biphasic with an initial lag time, where only minor changes in molecular weight are measured, and a terminal phase characterized by an accelerated polymer degradation. The three polymer type microspheres, i.e. PLGA50:50, PLGA75:25 and PLA, differ greatly in both the lag time and in the rate of terminal degradation. In the terminal phase an exponential decrease in molecular weight, as indicated by the linear alignment of the last three or four data points of each profile in the semilogarithmic plot is clearly shown. PLGA50:50 undergoes the fastest hydrolysis with a decrease in weight average molecular weight below 1000 within 35 days. Incidentally, as oligomers with a molecular weight below 1000 are water soluble, a $\bar{M}_w$-value of about 1000 or less is considered here as 'complete' polymer degradation. Furthermore, polymer degradation was characterized by weight average $\bar{M}_w$ rather than by the number average molecular weight. $\bar{M}_w$ was preferred because tailing in the polymer peak due to low molecular weight fragments renders setting of proper integration marks difficult and, hence, would affect greatly the calculation of number average molecular weight [37]. PLGA75:25 microspheres showed a lag time of 28 days, with only a 30% decrease in $\bar{M}_w$, followed by the exponential phase between days 28 and 56. After 8 weeks, $\bar{M}_w$ was below 10% of the initial $\bar{M}_w$. Finally, PLA microspheres required more than 3 months for complete erosion. This degradation profile was characterized by a lag time of 2 months, followed by accelerated polymer degradation up to day 100. Therefore, with increasing hydrophobicity of the polymers, the initial lag times became longer and the terminal exponential degradation rate lower.

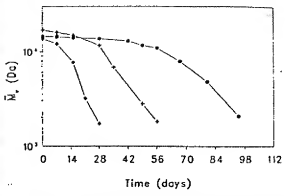


Fig. 1. Time-dependent $\bar{M}_w$ change of microspheres prepared with different types of polymers showing the effect of polymer composition: PLGA50:50-14 kDa (◇), PLGA75:25-17 kDa (+), PLA-14 kDa (●).

In addition to polymer composition, the initial $\bar{M}_w$ of the polymeric microspheres influenced substantially the total degradation time, although the overall degradation pattern followed a similar type of kinetics for the low and the high molecular weight particles, e.g. PLGA50:50-14 kDa versus PLGA50:50-35 kDa, PLGA75:25-17 kDa versus PLGA75:25–69 kDa, and PLA-14 kDa versus PLA-130 kDa (Fig. 2). As expected, PLA-130 kDa

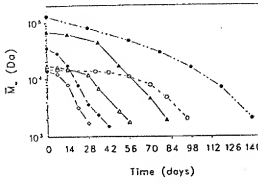


Fig. 2. Time-dependent $\bar{M}_w$ change of microspheres prepared with different types of polymers showing the effect of the initial molecular weight for various polymer compositions: PLGA50:50-14 kDa (◇), PLGA50:50-35 kDa (◇), PLGA75:25-17 kDa (△), PLGA75:25-69 kDa (△), PLA-14 kDa (○), PLA-130 kDa (●).

microspheres showed a very slow change in $\bar{M}_w$. Complete polymer erosion to water soluble oligomers was not finished up to day 140. Compared to the low molecular weight PLA-14 kDa, however, PLA-130 kDa hydrolysis was more pronounced during the initial part of the profile where no typical lag time could be detected. As a result of the sustained degradation, the polymeric microspheres studied were considered particularly useful for controlled antigen delivery over a period of several months. It was interesting to see that within the range of polymers studied an approx. 2-fold increase in $\bar{M}_w$ prolonged the time of degradation by 2 weeks (PLGA50:50-14 kDa versus PLGA50:50-35 kDa), at a 4-fold increase in $\bar{M}_w$ a prolongation of approx. 4 weeks (PLGA75:25-17 kDa versus PLGA75:25–69 kDa), and at a 9-fold increase in $\bar{M}_w$ a prolongation of degradation by approx. 6 weeks (PLA-14 kDa versus PLA-130 kDa) could be observed.

3.2. Morphology and size of the microspheres

It is reasonable to assume that surface morphology and, even more, microsphere size may play a crucial role for antigen delivery and presentation. Therefore, two different classes of microsphere sizes, one in the range of approximately 1–10 μm and the other of 10–100 μm, were prepared by the two preparation methods of spray-drying and coacervation, respectively (Fig. 3). Spray-drying produced smaller microspheres with the majority of particles below 5 μm, and coacervation yielded coarser microspheres, with the size distribution being a function of polymer $\bar{M}_w$ and of process parameters [34]. In general, the low $\bar{M}_w$ polymers gave a microsphere size in the range of 8 to 60 μm, whereas those of high $\bar{M}_w$ (PLGA75:25–69 kDa and PLA-130 kDa) ranged from 10 to 90 μm (Fig. 3). It is noteworthy that particle size distributions determined by laser light scattering and from SEM micrographs were in good agreement. Spray-dried microspheres exhibited a smooth and non-porous surface. In the case of coacervated microspheres, a similarly smooth surface morphology was detected when the antigen was microencapsulated as particulate material. On the opposite, when the antigen was entrapped as aqueous solution, a more porous surface was revealed on the SEM-micrographs.

Clearly, the use of the two different preparation techniques allowed microspheres with a wide range of polymer molecular weights and two distinct classes of particle sizes to be obtained. Both features are considered of prime importance for parenteral vaccine delivery systems.

3.3. Antigen microencapsulation and in vitro release

On the basis of the results obtained from polymer degradation and particle size distribution, the two antigens P30B2 and TT were microencapsulated using low molecular weight PLGA50:50-14 kDa and PLGA75:25-17 kDa, and a high molecular weight PLA-130 kDa. Both spray-drying (SD) and coacervation (CO) were applied. The various preparations are summarized in Table 1. For purely practical reasons, antigen powders rather than solutions were used in coacervation of PLGA75:25-17 kDa and PLA-130 kDa. It appears though, that the particulate antigens were less efficiently microencapsulated than the corresponding aqueous solutions. This difference is more significant with the synthetic antigen (27% for CO- PLGA75:25-17 kDa versus 57% for CO-PLGA50:50-14 kDa). It also appears that the efficiency to encapsulate the synthetic antigen is lower than for the natural high molecular weight TT.

Comparing the two encapsulation techniques, no

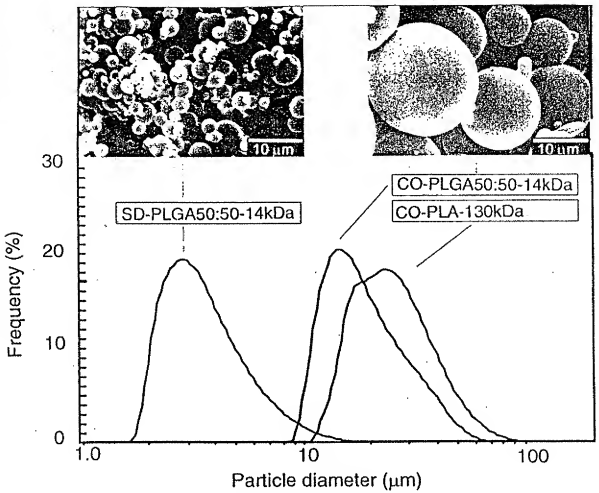


Fig. 3. SEM micrographs and particle size number distribution plots of typical microspheres prepared by spray-drying (SD-PLGA50:50-14 kDa) and by coacervation (CO-PLGA50:50-14 kDa, and CO-PLA-130 kDa).

significant difference in entrapment efficiency is observed if the antigens are processed in solution. Given the different preparation techniques, i.e. SD and CO, with the antigen for microencapsulation being in particulate form and in solution, an affect of the polymer composition on loading efficiency cannot be revealed unambiguously. Finally, with a few exceptions only, the loading efficiencies attained were satisfactory, and the actual loadings allowed to inject between 5 and 15 mg of P30B2-microspheres and between 1 and 4 mg of TT-microspheres per animal in the immunization studies.

Antigen release kinetics is considered one of the key parameters for vaccine delivery systems. Pulsatile release was actually achieved in the release of the 16 kDa P30B2 from the PLGA50:50-14 kDa microspheres (Fig. 4). After an initial burst release, which occurred during the first 1–2 days, a latency period of about 2 weeks was observed with very little antigen release. Release was completed after 7 weeks. The two release profiles obtained from the PLGA50:50-14 kDa microspheres prepared by spray-drying and by coacervation were very similar. For the coacervated microspheres, the onset of the second release pulse was slightly shifted to the right. Clearly, the term release pulse in understood in this work in a broad sense. The coacervated microspheres also gave rise to a substantially higher burst (20% of

Table 1
Antigen loading of microspheres

Antigen[a]	Polymer[a]	Microencapsulation technique[a]	Physical state of antigen for microencapsulation[b]	Loading (μg/mg microspheres)	Loading efficiency (%)[c]
P30B2	PLGA50:50	SD	Solution	3.51	54
	PLGA50:50	CO	Solution	6.00	57
	PLGA75:25	SD	Solution	6.59	60
	PLGA75:25	CO	Powder	2.06	27
	PLA	CO	Powder	2.64	26
TT	PLGA50:50	SD	Solution	18.20	90
	PLGA50:50	CO	Solution	8.50	85
	PLGA75:25	SD	Solution	21.50	100
	PLGA75:25	CO	Powder	7.00	70
	PLA	CO	Powder	5.80	58

[a]Abbreviations used are described in the experimental section; $\bar{M}_w$ of PLGA50:50 is 14 kDa, $\bar{M}_w$ of PLGA75:25 is 17 kDa and $\bar{M}_w$ of PLA is 130 kDa.
[b]The antigens were microencapsulated either as a 1–2% (w/v) aqueous solution or as a milled powder with particle size < 20 μm.
[c]The loading efficiency is defined as the percentage of the actual loading with respect to the theoretical loading.

the actual dose), than the spray-dried microspheres (10% of the actual dose). This difference may be explained by the slightly porous morphology of this particular MS-preparation (see under Section 3.2). It is also noteworthy that the total amount of P30B2 released from the coacervated PLGA50:50-14 kDa microspheres exceeded the measured loading by about 20%. This indicates that the recovery by the filtration method used to extract this antigen was maximal 80% with this particular preparation. The higher burst release and the underestimated loading of the coacervated PLGA50:50-14 kDa microspheres must have both contributed to the fact that this preparation exhibits an area under the second release pulse similar to the spray-dried PLGA50:50-14 kDa, although the actual loading of the coacervated microspheres exceeded that of the spray-dried product by about 40%.

While the microencapsulation method essentially affected the extent of the burst release, the polymer composition greatly determined the overall release kinetics, particularly the onset and duration of the second release pulse (Fig. 5). As mentioned before,

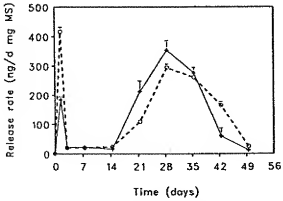


Fig. 4. In vitro release rates (in ng antigen per day and per mg microspheres) of P30 B2 from spray-dried (+) and coacervated (O) PLGA50:50-14 kDa microspheres. Error bars indicate standard deviations with $n = 3$.

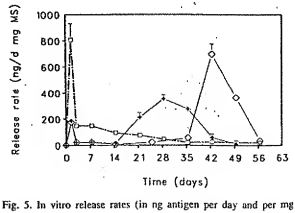


Fig. 5. In vitro release rates (in ng antigen per day and per mg microspheres) of P30B2 from spray-dried PLGA50:50-14 kDa (+) and PLGA75:25-17 kDa (◇), and from coacervated PLA-130 kDa (□) microspheres. Error bars indicate standard deviations with $n = 3$.

the onset and maximum of the second pulse for the PLGA50:50-14 kDa appear around day 14 and day 28, respectively, while for PLGA75:25-17 kDa, these two time points are shifted to day 35 and day 43. In contrast, for the slowly degrading PLA-130 kDa, no additional release pulse was detected within the time period studied. For both the PLGA50:50-14 kDa and PLGA75:25-17 kDa microspheres, the onset of the second pulse corresponded to a $\bar{M}_w$ of about 7000 to 8000 Da during the in vitro polymer degradation, while the maximum release rate was measured at a time point corresponding to a $\bar{M}_w$ of about 2000 to 4000 Da. From the polymer degradation profiles, the second release pulse from the PLA-130 kDa microspheres was expected after about 4 months, though the experiment was stopped before that time point.

Similarly to P30B2, TT release was not primarily affected by the preparation method and, hence, by the size of the microspheres, but by the type of polymer (Fig. 6). While the onset and duration of the second TT pulse from PLGA50:50-14 kDa microspheres was virtually identical to that observed for P30B2 with this same polymer, this similarity was not found for the PLGA75:25-17 kDa microspheres. For TT, the second release pulse from PLGA75:25-17 kDa microspheres started on day 56 and ended on day 84, corresponding to a shift of approximately 3 weeks with respect to P30B2. When comparing TT release profiles to microsphere degradation, a close correlation between the second release pulse and the final stage of polymer hydrolysis was observed. Clearly, the second TT pulse started when microsphere degradation reached a $\bar{M}_w$ of 2000 to 3000 Da. Although a $\bar{M}_w$ of 1000 Da was considered as complete polymer erosion, TT release was not yet finished at this time point but continued for an additional 4 weeks. For TT, the total amount released and the quantity determined as loading were in close agreement, indicating that the recovery of the extraction method used was in the order of 90–100% for this large protein.

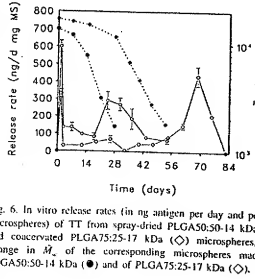


Fig. 6. In vitro release rates (in ng antigen per day and per mg microspheres) of TT from spray-dried PLGA50:50-14 kDa (○) and coacervated PLGA75:25-17 kDa (◇) microspheres, and change in $\bar{M}_w$ of the corresponding microspheres made of PLGA50:50-14 kDa (●) and of PLGA75:25-17 kDa (◇).

The excellent correlation between the second release pulse and the polymer degradation time for both antigens may indicate the feasibility to design PLA/PLGA-microspheres with well defined pulses at any desired time point, e.g. after 1, 2, 3, 6 months etc., corresponding to the degradation profiles in Fig. 2. However, although the onset and maximum of the second release pulse were well defined, the duration of the pulse extended over 2–4 weeks.

3.4. Immune response

Anti-TT and anti-P30B2 antibody responses were determined for all of the experimental groups at intervals of 2–4 weeks, up to week 45 for TT and week 28 for P30B2. In this contribution, the immunological response is expressed in terms of antibody titers.

For the synthetic and weakly immunogenic P30B2, the microsphere size did not appear to affect the general time course of antibody titers, although a slightly slower increase of the titers was observed with the larger size particles prepared by coacervation (Fig. 7). After 6 weeks, however, the two microsphere preparations showed very similar profiles. A comparison with the profile obtained with the IFA-formulation indicated a kinetic difference in the immune response. With IFA, the antibody level approached the maximum 4–6 weeks after immunization and started to decrease significantly after 4 months. With the microspheres, however, the antibody levels remained elevated over the entire time period studied (28 weeks).

The effect of the polymer type on anti-P30B2 specific antibody levels is presented in Fig. 8. Here again, significant differences between the profiles

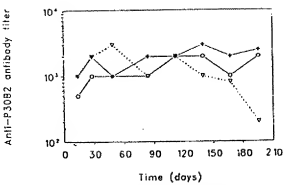


Fig. 7. Time course of anti-P30B2 antibody response in BALB/c mice upon injection of 50 μg P30B2 in different formulations showing the effect of the microencapsulation method and the related particle size: spray-dried (+) and coacervated (O) PLGA50:50-14 kDa microspheres, and reference formulation IFA (▽). Individual curves represent the geometric mean of antibody titers obtained from each group by ELISA.

were noticed in the early stage of the immune response, i.e. during the first 3 months. The fast degrading small-sized microspheres (SD-PLGA50:50-14 kDa) gave rise to an immediate and strong antibody response comparable to IFA. By contrast, the slower degrading PLGA75:25 microspheres (SD-PLGA75:25-17 kDa) and, even more, the larger size CO-PLA-130 kDa microspheres produced a more gradual increase in antibody titers with

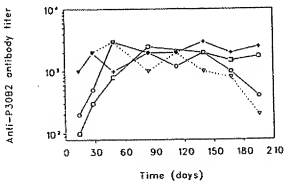


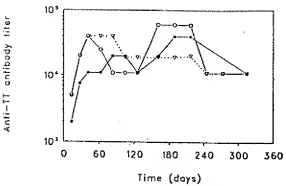
Fig. 8. Time course of anti-P30B2 antibody response in BALB/c mice upon injection of 50 μg P30B2 in different formulations showing the effect of the polymer type and the related release characteristics: spray-dried PLGA50:50-14 kDa (+), spray-dried PLGA75:25-17 kDa (O) and coacervated PLA-130 kDa (□) microspheres, and reference formulation IFA (▽). Individual curves represent the geometric mean of antibody titers obtained from each group by ELISA.

the maximum reached only 6 weeks (for SD PLGA75:25-17 kDa) or 12 weeks (for CO-PLA-13(kDa) after immunization. While for the PLGA50:5(14 kDa and the PLA-130 kDa microspheres th antibody levels remained elevated over the entir period studied, they tended to decrease after 1! weeks postimmunization in the case of PLGA75:25 17 kDa, similarly to those measured in the IFA group. Clearly, the expected release pulse from th PLA polymer after approximately 4 months eithe did not materialize in vivo or had no effect on th antibody titer. For the PLGA50:50-14 kDa an PLGA75:25-17 kDa microspheres, the determine release pulse was possibly too close to the initi burst release to exert an influence on the antibod titers. In mice, no booster effect could be observe with any of the microsphere preparations after singl injection, which contrasts the data of the in vitr experiments.

The data of the immune response unambiguous! demonstrated the immunostimulating properties (all the microsphere preparations. The observed di ferences between the various formulations wer essentially limited to the kinetics of the early stag antibody response. Larger and slowly degradir microspheres induced antibody titers more slow than the small-sized and fast degrading micr spheres.

In contrast to the synthetic P30B2-formulation the TT microspheres generated more distinct kinet antibody profiles. Typically, the PLGA75:25-17 kD microspheres produced a vague anti-TT boost effect between days 120 and 180 (Fig. 9). In th initial stage of the profiles, a significantly fast antibody response was measured in the group of th small-sized microspheres (SD-PLGA75:25-17 kD. On the other side, the maximum antibody titer an duration of the response did not appear to l influenced by the particle size (SD- PLGA75:25-1 kDa versus CO-PLGA75:25-17 kDa). The micr sphere formulations elicited antibody responses cor parable to those obtained with the convention vaccine (TT-alum).

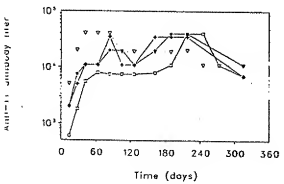
The influence of polymer composition on t antibody response is illustrated in Fig. 10, wi large-sized microspheres prepared by coacervati No difference was observed between the two typ of copolymers, the PLGA50:50 and PLGA75:25.



g. 9. Time course of anti-TT antibody response in BALB/c ice upon injection of 20 μg TT in different formulations owing the effect of the microencapsulation method and the lated particle size: spray-dried (○) and coacervated (●) LGA75:25-17 kDa microspheres, and reference formulation um (▽). Individual curves represent the geometric mean of ntibody titers obtained from each group by ELISA.

ery slight boosting effect was also noticeable with reparation PLGA50:50-14 kDa, as pointed out ready for the PLGA75:25 microspheres. With the owly degrading and releasing PLA microspheres, ntibody titers increased much slower and reached e maximum level only after about 220 days. Again, slight booster effect occurred between days 180 id 220.

Generally, all TT microsphere formulations eli-



. 10. Time course of anti-TT antibody response in BALB/c e upon injection of 20 μg TT in different formulations wing the effect of the polymer type and the related release racteristics: coacervated PLGA50:50-14 kDa (+), GA75:25-17 kDa (●) and PLA-130 kDa (□) microspheres, l reference formulation alum (▽). Individual curves represent geometric mean of antibody titers obtained from each group ELISA.

cited a high antibody response comparable to the one obtained with the TT adsorbed on alum, and antibody levels remained elevated over a long period of time. Significant differences in antibody levels were observed only in the early stage of the immunological response, but not at later times. The differences in antibody titers in the initial phase can be explained by both the two classes of particle sizes used, and by the different in vitro antigen release kinetics in combination with polymer degradation. The small-sized microspheres gave generally faster antibody response than the larger microspheres (Fig. 9), and the slower initial TT-release from PLA-130 kDa as compared to PLGA50:50-14 kDa and PLGA75:25-17 kDa is reflected by a slower rise in antibody levels (Fig. 10).

4. Discussion

To rationalize the design of antigen delivery microspheres, this study aimed at a better understanding of the correlation between relevant microsphere characteristics, such as degradation time, particle size and release profile, on the one side, and the time course of the immune response, on the other side. Although an increasing number of studies on antigens microencapsulated into biodegradable microspheres have been published over the past 5 years, only very few have made attempts to investigate the influence of relevant microsphere characteristics on the immunological response [1,30,31].

The possibility of controlling antigen release through the degradation time of polymeric microspheres represents an attractive feature for antigen delivery. As polyester degradation mechanisms and time depend on processing conditions and sample dimensions, data obtained from native polymer powder or particular forms of implants are not necessarily representative for microsphere erosion [24]. Therefore, an in vitro degradation study was conducted with a series of PLA- and PLGA-based microspheres prepared by coacervation. The degradation profiles showed a uniform and consistent pattern revealing the importance of polymer hydrophobicity and molecular weight (Fig. 1 and Fig. 2). An increasing number of glycolide units renders the polymer more hydrophilic, resulting in a more

pronounced water uptake [21,27,38]. With increasing hydration, the glass transition temperature, T_g, decreases below the incubation temperature of 37°C, shifting the PLGA chains into a rubbery state and facilitating hydrolytic cleavage [18]. Polyester hydrolysis was reported to occur prior to polymer mass loss, whereby, in general, the change in $\bar{M}_w$ follows first-order kinetics [25]. Our data suggest that such an exponential decrease is not observed from the very beginning but follows a lag period of several days to several weeks with little change in $\bar{M}_w$ occurring. For the low and intermediate molecular weight microspheres, this initial phase primarily depends on the type and hydrophilicity of the polymer. A more pronounced change in $\bar{M}_w$ in the first few weeks was noticed though for the high molecular weight PLA. This finding is consistent with the reported observation of a faster degradation rate in high molecular weight polymers [21]. The different degradation kinetics between PLA-14 kDa and PLA-130 kDa might be explained by the substantial amount of residual solvents present in the PLA-130 kDa (3% DCM and 5% OMCTS), as compared to PLA-14 kDa microspheres (0.2% DCM and 1% OMCTS), contributing to a decrease of T_g and, hence, facilitating water uptake into the partially rubbery polymer bulk [34].

The accelerated degradation rate in the terminal stage of the profiles is in agreement with other reports and has been explained by the presence of increasing amounts of acidic degradation products or of Na^+ ions from the buffer [16,23,28,39]. More specifically, autocaalysis by acidic oligomers accumulated in the polymer bulk due to their reduced diffusion and dissolution characteristics at low pH has been suggested [25]. In our degradation experiments, the medium was regularly replaced by fresh buffer. Nevertheless, in the later stage of degradation, the buffer capacity was not sufficient to stabilize the pH around 7. In a recent study, unambiguous evidence was provided that the pH lowered by degradation products altered the degradation kinetics of microspheres [28]. This fact was illustrated by the large differences in the erosion profiles between particles kept either at constant pH in a dialysis bag or under non-constant pH conditions in buffered saline, becoming increasingly acidic through the accumulation of degradation products.

Knowledge of the polymer degradation tim enabled the selection of appropriate polymers f antigen microencapsulation, which were expected provide release pulses after 1, 2 and 4–6 montl The striking concurrence between the polymer degr dation time (Fig. 1 and Fig. 2) and the secon antigen release pulse of both antigens studied (Fig 4–6) is of prime importance for programming p tential booster doses from this delivery system. Tl possible mechanisms responsible for the pulsati release behavior of peptides and proteins from PL/ PLGA microspheres have been discussed by seve authors [13,26,27,40]. The three distinct phases protein release observed have been described as t the initial burst release with the diffusion of acti compound located near the microsphere surfa during polymer hydration, (ii) the latency phase wi inhibited diffusion, and (iii) the release pulse due biodegradation and polymer mass erosion. It generally assumed that in the latent phase some ty of interactions between the negatively charged pol meric carboxyl groups and the protein must reta the active compound. Specifically, an ion exchan mechanism has been suggested [27]. If it is inde this mechanism which is mainly controlling t release of the actual antigens, the timely differen in the appearance of the second release pulse b tween TT and P30B2 might be explained by th molecular weight difference. The larger TT (1 kDa) must exhibit, at a given pH, a higher density ammonium groups able to interact with the pol meric carboxyl groups, as compared to the 16 kl P30B2. This would indeed result in a strong interaction with the larger TT. The formation of ionic complex between the carboxyl group carryi polymer and the protonated protein is, in gener expected within the approximate pH-range of 3. 5.5, close to or below the pI of the protein (e.g. pI TT = 5.1), and above the pK_a of the glycolic a lactic acids. In the case of charge separation on t protein, reaction with the anionic oligomers may al occur at higher pH. With respect to polymer degr dation, this may signify that proteins of a small size or of a lower pI will dissolve from the ion oligomer-protein complex at an earlier stage th larger proteins.

This interpretation of the latency phase observ in this study suggests a pH-microenvironment insi

ie polymer particles with a lower pH than that of he release medium. Such an acidic microenviron- ient has also been suggested by other authors 16,29]. The generation of high amounts of acidic legradation products inside the microspheres or in 'ie incubation medium has caused well founded oncerns about the stability of unreleased and re- ased proteins [4,16,28,29]. With the analytical ethods used here, no degradation of the two ntigens could be detected. This does not mean, owever, that alteration of antigenicity or even nmunogenicity may be excluded during the release. hus, a much more complete set of analytical ethods would have to be used to detect degradation levant for the biological activity. Previous studies y our group and by others have focused on TT- ability in aqueous solutions and under freeze-dry- g conditions [4,41]. Presently, several groups are vestigating intensively TT stability within biode- adable microspheres. As TT is not likely to drolyze under the actual release conditions, co- lent intermolecular bonding or simple physical gregation between TT molecules or between TT id oligomers are also considered.

In addition to the pulsatile antigen release, the rticle size is generally expected to influence the mune response. It was shown that small-sized rticles, i.e. $<10\ \mu m$, are very efficiently taken up macrophages resulting in improved antigen pre- ntation and enhanced adjuvant effect [42,43]. Our ta emphasize that the particle size, within the ige tested, exerts no effect on the maximum tibody titers nor on the duration of the response. e observed, however, that small microspheres, e.g.)-PLGA50:50-14 kDa, elicited 5- to 10-fold higher tibody titers at early time points than those de- ted with larger microspheres. Subsequently, anti- dy titers in the large-sized microsphere groups gan to rise so that no significant difference existed tween small and larger microspheres after several nths (Figs. 9 and 10), which is in agreement with ier studies [43].

The ability of a single injection of microspheres to vide distinct and timely release pulses of mi- encapsulated antigen may greatly facilitate future cine development. The antibody response ob- ted with different formulations during the time rval tested reflects, to some extent, the release properties of the microspheres. However, the release pulses measured in vitro induce only a vague booster effect in mice. It is not clear yet if this is a consequence of an inappropriate onset of the release pulses, the extended duration of the pulses, or the loss of immunogenicity of the antigens at later time points. To our knowledge, no unambiguous booster effect after single parenteral administration has been shown so far in similar type of investigations [2,30]. Considering all of the individual microsphere preparations, no essential differences were observed in the maximum antibody responses. In a previous study by our group, we have shown that the use of microsphere mixtures consisting of different types of polymeric microspheres produce substantially stronger immune responses than the individual microsphere types [36]. Besides the very high and sustained antibody titers, T-cell proliferative responses were greatly enhanced in the groups of mice receiving a single injection of a microsphere mixture. Clearly, 45 weeks after immunization, the proliferative response of the microsphere mixture group and the group having received three injections of alum adsorbed TT (at weeks 0, 5 and 16) was comparable. Although the microsphere mixtures elicited greatly enhanced and more prolonged immune responses, there was no indication of a booster effect. This might indicate that the overlapping of the release pulses from the different individual microspheres types may not only enhance the maximum response, but may hide simultaneously any booster effects.

5. Conclusions

With the two antigens studied, the natural tetanus toxoid (TT) and a weakly immunogenic malaria peptide (P30B2), a clear correlation between the in vitro polymer degradation and the in vitro antigen release profile was found. Antigen release follows a pulsatile pattern with a first pulse representing the so-called burst effect and a second pulse determined by the polymer degradation time. The antigen containing microspheres exerted an important immunostimulating effect in terms of antibody response. Antibody titers remained elevated over the entire time period studied, i.e. up to 45 weeks, and were comparable to the P30B2 in IFA and TT on alum.

However, the pulsatile in vitro release profile generated only a slight boosting effect in vivo. Nonetheless, the time course in antibody titers could be explained, at least partly, by the particle size and the release behaviour. A slower increase in antibody titers was observed with the microspheres of larger size or exhibiting a slower release. Immediate and highest antibody levels were elicited with the small-sized fast degrading microspheres.

Acknowledgments

This work was supported by grants from WHO and the Swiss National Foundation for Scientific Research (Grant No. 31-37440.93).

References

[1] M.J. Alonso, S. Cohen, T.G. Park, R.K. Gupta, G.R. Siber and R. Langer, Determinants of release rate of tetanus vaccine from polyester microspheres, Pharm. Res. 10 (1993) 945–953.

[2] M.J. Alonso, R.K. Gupta, C. Min, G.R. Siber and R. Langer, Biodegradable microspheres as controlled-release tetanus toxoid delivery systems, Vaccine 12 (1994) 299–306.

[3] Y. Men, G. Corradin, C. Thomasin, H.P. Merkle and B. Gander, Immunopotentiation of a synthetic antigen by incorporation into biodegradable microspheres, Proc. Int. Symp. Control. Rel. Bioact. Mater. 21 (1994) 50–51.

[4] B. Gander, C. Thomasin, H.P. Merkle, Y. Men and G. Corradin, Pulsed tetanus toxoid release from PLGA-microspheres and its relevance for immunogenicity in mice, Proc. Int. Symp. Control. Rel. Bioact. Mater. 20 (1993) 65–66.

[5] M.T. Aguado and P.-H. Lambert, Controlled release vaccines — Biodegradable polylactide/polyglycolide (PL/PG) microspheres as antigen vehicles, Immunobiology 184 (1992) 113–125.

[6] M.T. Aguado, Future approaches to vaccine development: Single-dose vaccines using controlled-release delivery systems, Vaccine 11 (1993) 596–597.

[7] J.H. Eldridge, C.J. Hammond, J.A. Meulbroek, J.K. Staas, R.M. Gilley and T.R. Tice, Controlled vaccine release in the gut-associated lymphoid tissues. I. Orally administered biodegradable microspheres target the Peyer's patches, J. Control. Release 11 (1990) 205–214.

[8] D.T. O'Hagan, Oral immunization and the common mucosal immune system, in: D.T. O'Hagan (Ed.), Novel Delivery Systems for Oral Vaccines, CRC Press, Boca Raton, FL, 1994, pp. 1–24.

[9] J. Mestecky, Z. Moldoveanu, M. Novak, W.-Q. Huang, R.S. Gilley, J.K. Staas, D. Schafer and R.W. Compans, Biodegradable microspheres for the delivery of oral vaccines, Control. Release 28 (1994) 131–141.

[10] A.J. Almeida, H.O. Alpar and M.R.W. Brown, Immune response to nasal delivery of antigenically intact tetanus toxoid associated with poly(L-lactic acid) microspheres in rats, rabbits and guinea-pigs, J. Pharm. Pharmacol. 45 (1993) 198–203.

[11] J. Preis and R.S. Langer, A single-step immunization by sustained antigen release, J. Immunol. Methods 28 (1979) 193–197.

[12] J. Kohn, S.M. Niemi, E.C. Albert, J.C. Murphy, R. Langer and J.G. Fox, Single-step immunization using a controlled release biodegradable polymer with sustained adjuvant activity, J. Immunol. Methods 95 (1986) 31–38.

[13] L.M. Sanders, J.S. Kent, G.I. McRae, B.H. Vickery, T. Tice and D.H. Lewis, Controlled release of luteinizing hormone-releasing hormone analogue from poly(d,l-lactic co-glycolide) microspheres, J. Pharm. Sci. 73 (1984) 1294–1297.

[14] G.E. Visscher, R.L. Robison, H.V. Maulding, J.W. Fong, J. Pearson and G.J. Argentieri, Biodegradation of and tissue reaction to 50:50 poly(DL-lactide-co-glycolide) microcapsules, J. Biomed. Mater. Res. 19 (1985) 349–365.

[15] G.E. Visscher, R.L. Robison, H.V. Maulding, J.W. Fong, J. Pearson and G.J. Argentieri, Biodegradation of and tissue reaction to poly(DL-lactide) microcapsules, J. Biomed. Mater. Res. 20 (1986) 667–676.

[16] R.A. Kenley, M.O. Lee, T.R. Mahoney II and L.M. Sanders, Poly(lactide-co-glycolide) decomposition kinetics in vivo and in vitro, Macromolecules 20 (1987) 2398–2403.

[17] H.T. Wang, H. Palmer, R.J. Linhardt, D.R. Flanagan and E. Schmitt, Degradation of poly(ester) microspheres, Biomaterials 11 (1990) 679–685.

[18] S.S. Shah, Y. Cha and C.G. Pitt, Poly(glycolic acid-co-DL-lactic acid): Diffusion or degradation controlled drug delivery? J. Control. Release 18 (1992) 261–270.

[19] G. Rafler and M. Jobmann, Controlled release systems of biodegradable polymers. 1st communication: Hydrolytic degradation of biodegradable aliphatic polyesters, Pharm. Ind. 56 (1994) 565–570.

[20] T.G. Park, Degradation of poly(D,L-lactic acid) microspheres: effect of molecular weight, J. Control. Release 30 (1994) 161–173.

[21] D.T. O'Hagan, H. Jeffery and S.S. Davis, The preparation and characterization of poly(lactide-co-glycolide) microspheres: III Microparticle/polymer degradation rates and the in vitro release of a model protein, Int. J. Pharm. 103 (1994) 37–45.

[22] S. Cohen, T. Yoshioka, M. Lucarelli, L.H. Hwang and R. Langer, Controlled delivery systems for proteins based on poly(lactic/glycolic acid) microspheres, Pharm. Res. 8 (1991) 713–720.

[23] E.A. Schmitt, D.R. Flanagan and R.J. Linhardt, Degradation and release properties of pellets fabricated from the commercial poly(D,L-lactide-co-glycolide) biodegradable polymers, J. Pharm. Sci. 82 (1993) 326–329.

[24] M. Vert, S. Li and H. Garreau, More about the degradation of LA/GA-derived matrices in aqueous media, J. Control. Release 16 (1991) 15–26.

[25] C.G. Pitt, Non-microbial degradation of polyesters: Mechanisms and modifications, in: M. Vert, J. Feijen, A. Albertson, G. Scott and E. Chiellini (Eds.), Biodegradable Polymers and Plastics, Redwood Press, 1992, pp. 7–17.

[26] J.R. Lawter, N.S. Brizzolara, M.G. Lanzilotti and G.O. Morton, Drug release from poly(glycolid-co-lactide) microcapsules, Proc. Int. Symp. Control. Rel. Bioact. Mater. 14 (1987) 99–100.

[27] D. Bodmer, T. Kissel and E. Traechslin, Factors influencing the release of peptides and proteins from biodegradable parenteral depot systems, J. Control. Release 21 (1992) 129–138.

[28] T.G. Park, W. Lu and G. Crotts, Importance of in vitro experimental conditions on protein release kinetics, stability and polymer degradation in protein encapsulated poly(D,L-lactic acid-co-glycolic acid) microspheres, J. Control. Release 33 (1995) 211–222.

[29] W. Lu and T.G. Park, Protein release from poly(lactic-co-glycolic acid) microspheres: Protein stability problems, PDA J. Pharm. Sci. Technol. 49 (1995) 13–19.

[30] I. Esparza and T. Kissel, Parameters affecting the immunogenicity of microencapsulated tetanus toxoid, Vaccine 10 (1992) 714–720.

[31] M. Singh, A. Singh and G.P. Talwar, Controlled delivery of diphtheria toxoid using biodegradable poly(D,L-lactide) microcapsules, Pharm. Res. 8 (1991) 958–961.

[32] D. Valmori, A. Pessi, E. Bianchi, G. Corradin, Use of human universally antigenic tetanus toxin T cell epitopes as carriers for human vaccination, J. Immunol. 149 (1992) 717–721.

[33] B. Gander, E. Wehrli, R. Alder and H.P. Merkle, Quality improvement of spray-dried, protein-loaded D,L-PLA microspheres by appropriate polymer solvent selection, J. Microencapsulation 12 (1995) 83–97.

[34] C. Thomasin, P. Johansen, R. Alder, R. Bemsel, G. Hottinger, H. Altorfer, A.D. Wright, E. Wehrli, H.P. Merkle and B. Gander, Approaching the problem of residual solvents in biodegradable microspheres prepared by coacervation, Eur. J. Pharm. Biopharm. 42 (1996) in press.

[35] D.W. Van Krevelen, Properties of Polymers, 3rd Ed., Elsevier, Amsterdam, The Netherlands, 1990.

[36] Y. Men, C. Thomasin, H.P. Merkle, B. Gander and G. Corradin, A single administration of tetanus toxoid in biodegradable microspheres elicits T cell and antibody responses similar or superior to those obtained with aluminum hydroxide, Vaccine 13 (1995) in press.

[37] H. Pistner, D. Bendix, J. Muhling and J. Reuther, Poly(L-lactide): A long term degradation study in vivo, Part III. Analytical characterization, Biomaterials 14 (1993) 291–298.

[38] R.L. Dunn, J.P. English, J.D. Strobel, D.R. Cowsar and T.R. Tice, Preparation and evaluation of lactide/glycolide copolymers for drug delivery, in: C. Migliaresi (Ed.), Polymers in Medicine III, Elsevier, Amsterdam, 1988.

[39] K. Makino, H. Ohshima and T. Kondo, Mechanism of hydrolytic degradation of poly(L-lactide) microcapsules: effects of pH, ionic strength and buffer concentration, J. Microencapsulation 3 (1986) 203–212.

[40] M.S. Hora, R.K. Rana, J.H. Nunberg, T.R. Tice, R.M. Gilley and M.E. Hudson, Release of human serum albumin from poly(lactide-co-glycolide) microspheres, Pharm. Res. 7 (1990) 1190–1194.

[41] S.P. Schwendeman, J.H. Lee, R.K. Gupta, H.R. Costantino, G.R. Siber and R. Langer, Inhibition of moisture-induced aggregation of tetanus toxoid by protecting thiol groups, Proc. Int. Symp. Control. Rel. Bioact. Mater. 21 (1994) 54–55.

[42] Y. Tabata and Y. Ikada, Effect of the size and surface charge of polymer microspheres on their phagocytosis by macrophage, Biomaterials 9 (1988) 356–362.

[43] J.K. Staas, J.H. Eldridge, D.J. Morgan, O.B. Finch, T.R. Tice and R.M. Gilley, Microsphere vaccines: enhanced immune response through adjuvant effect and multiple-pulse release capability, Proc. Int. Symp. Control. Rel. Bioact. Mater. 18 (1991) 619–620.

[44] S. Bennett and E.M. Riley, The statistical analysis of data from immunoepidemiological studies, J. Immunol. Methods 146 (1992) 229–239.